

# Distinct Functions of T- and L-Type Calcium Channels during Activation of Bovine Adrenal Glomerulosa Cells\*

MICHEL F. ROSSIER†, MURIEL M. BURNAY, MICHEL B. VALLOTTON, AND ALESSANDRO M. CAPPONI

Division of Endocrinology and Diabetology, University Hospital, Geneva, Switzerland

## ABSTRACT

Calcium influx into adrenal glomerulosa cells is a key event during the stimulation of aldosterone secretion by physiological increases in extracellular potassium concentrations. Two types of voltage-operated calcium channels, T- and L-types, are present on bovine glomerulosa cells, but their respective functions are not yet clearly defined. Using the patch-clamp method in the perforated patch configuration combined with microfluorimetry of cytosolic calcium, we demonstrate that L-type channels are exclusively responsible for the sustained elevation of cytosolic calcium observed upon stimulation with extracellular potassium, even at low, physiological concentrations of this agonist. In contrast, aldosterone secretion appears

closely related to T-type channel activity. Moreover, when the activity of each channel type is selectively modulated by pharmacological agents, such as dihydropyridines or zonisamide, the cytosolic calcium response can be clearly dissociated from the steroidogenic response. Similarly, modulation of T channel activation by protein kinase C results in a parallel inhibition of aldosterone secretion, without any effect on the levels of cytosolic free calcium. This direct functional link between T-type calcium channel activity and steroidogenesis suggests a model in which calcium entering the cell through these channels bypasses the cytosol to activate intramitochondrial steps of aldosterone biosynthesis. (*Endocrinology* 137: 4817–4826, 1996)

THE STIMULATION of aldosterone synthesis in adrenal glomerulosa cells by angiotensin II (AngII) or potassium ion is maintained through a sustained influx of  $\text{Ca}^{2+}$  into the cell (1–3). Although a crucial role for  $\text{Ca}^{2+}$  entry has been recognized for many years (4), the nature of the pathways allowing extracellular  $\text{Ca}^{2+}$  to reach its intracellular target sites, such as the mitochondria, where the cation can control key steps of steroidogenesis, was only recently determined.

A biphasic response of the cytosolic free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_c$ ) can be clearly observed upon AngII stimulation; indeed, a first  $\text{Ca}^{2+}$  release phase from intracellular stores triggered by the formation of inositol 1,4,5-trisphosphate (5–7) is followed by a sustained  $\text{Ca}^{2+}$  influx. This second entry phase is mainly attributable to the activation of a capacitative influx resulting from the depletion of intracellular  $\text{Ca}^{2+}$  stores (8, 9); however, a component of this influx is also due to voltage-operated  $\text{Ca}^{2+}$  channels activated during AngII-induced cell depolarization.

In contrast, extracellular  $\text{K}^+$  does not affect intracellular  $\text{Ca}^{2+}$  pools and, therefore, appears to exclusively activate voltage-operated  $\text{Ca}^{2+}$  channels as a consequence of its depolarizing action. However, both L- and T-type  $\text{Ca}^{2+}$  channels are expressed in bovine glomerulosa cells (10, 11), and which channel is involved in the activation of glomerulosa cells by KCl is still a matter of debate. Whereas the slow kinetics of L-type channel inactivation obviously favor a role

of these channels during long lasting stimulations of steroidogenesis by  $\text{K}^+$ , the lower threshold of activation of T-type channels has been proposed to provide the cell with an exquisite sensitivity to low, physiological concentrations of extracellular  $\text{K}^+$ . Moreover, the existence of a window of voltage, which is compatible with the membrane potential values reached during stimulation with physiological concentrations of extracellular  $\text{K}^+$  and in which a statistical proportion of T-type channels can be maintained open (12, 13), supports the hypothesis of a role for the latter channels in cell activation.

Early pharmacological studies aiming at discriminating between T- and L-type channel function largely contributed to the present confusion, because of the low specificity and high concentrations of the agents used and the poor control of the action of these channel blockers on specific current amplitudes (2, 14). Additional problems could also originate from the fact that steroidogenesis and  $[\text{Ca}^{2+}]_c$  were not always systematically measured under the same conditions, and both parameters were generally considered *a priori* as inextricably related.

Recently, Barrett *et al.* (15) used the spider toxin  $\omega$ -agatoxin IIIA to specifically and completely block L-type  $\text{Ca}^{2+}$  channels in bovine glomerulosa cells. Under these conditions, the steroidogenic response to physiological concentrations of  $\text{K}^+$  (7 mM) remained unaffected, suggesting that aldosterone secretion is primarily stimulated by  $\text{Ca}^{2+}$  entry through T-type  $\text{Ca}^{2+}$  channels. Moreover, upon activation with a much higher concentration of  $\text{K}^+$  (60 mM), which is believed to efficiently open L-type channels, aldosterone production was even enhanced by  $\omega$ -Aga IIIA and inhibited by the L-type channel activator BayK 8644, an observation that has also been reported in rat cells (16). A negative modulation of

Received April 19, 1996.

Address all correspondence and requests for reprints to: Dr. Michel F. Rossier, Division of Endocrinology and Diabetology, University Hospital, CH-1211 Geneva 14, Switzerland. E-mail: rossier@cmu.unige.ch.

\* This work was supported by Swiss National Science Foundation Grants 32-39277.93 and 31-42178.94.

† Recipient of a grant from the Prof. Max Cloëtta Foundation.

steroidogenesis by  $\text{Ca}^{2+}$  when entering the cell through L-type channels was, therefore, proposed (15).

In the present study, we examined the effects of various pharmacological agents, such as dihydropyridines and zonisamide, on both T- and L-type channel activities as well as on  $[\text{Ca}^{2+}]_i$  and aldosterone production to determine which parameters are functionally linked upon stimulation with extracellular  $\text{K}^+$ . We found that whereas L-type channels are mostly responsible for the variations in  $[\text{Ca}^{2+}]_i$ , T-type, but not L-type, channel activity is closely correlated with aldosterone biosynthesis.

### Materials and Methods

Percoll was obtained from Pharmacia (Piscataway, NJ), and Cell-Tak from Inotech (Dottikon, Switzerland). Amphotericin B, nystatin, tetrodotoxin, sodium ATP, sodium GTP, ionomycin, EGTA, and nifedipine were purchased from Sigma (St. Louis, MO), and pluronic acid, fluo-3, and fura-2 acetoxymethyl esters were obtained from Molecular Probes (Eugene, OR). Phorbol 12-myristate 13-acetate (PMA) was purchased from LC Laboratories (Woburn, MA), and calciseptine was purchased from Latoxan (Rosans, France). BayK 8644 was kindly donated by Bayer (Leverkusen, Germany), and zonisamide was a generous gift from Dr. S. Kurooka, Dainippon Pharmaceutical Co. (Osaka, Japan).

### Adrenal glomerulosa cell isolation and culture

Bovine adrenal glands were obtained from a local slaughterhouse, and glomerulosa cells were prepared by enzymatic dispersion, purified on a Percoll density gradient, and maintained in culture for 2–4 days, as described in detail previously (11). The relatively small size of glomerulosa cells compared to that of fasciculata cells, the presence of lipid droplets, as well as the responsiveness to AngII confirmed that more than 95% of the cell population was composed of glomerulosa cells.

### Patch-clamp measurements

The activity of voltage-operated  $\text{Ca}^{2+}$  channels in bovine adrenal glomerulosa cells was recorded under voltage clamp either in the whole cell configuration of the patch clamp technique, as previously described (13), or in combination with  $[\text{Ca}^{2+}]_i$  measurement with the fluorescent dye fluo-3 in the perforated patch configuration (17). In the latter case, the bath solution contained 117 mM tetraethylammonium chloride, 20 mM  $\text{CaCl}_2$ , 0.5 mM  $\text{MgCl}_2$ , 5 mM D-glucose, 32 mM sucrose, and 200 nM tetrodotoxin and was buffered to pH 7.5 with 10 mM HEPES- $\text{CsOH}$ . The patch pipette (3–6 M $\Omega$ ; Clark 150T, Reading, UK) contained 130 mM  $\text{CsCl}$ , 5 mM  $\text{MgCl}_2$ , and 1 mM  $\text{CaCl}_2$ , and the pH was buffered to 7.2 with 20 mM HEPES- $\text{CsOH}$ . The pipette solution also contained 0.2 mg/ml nystatin (17) or 0.24 mg/ml amphotericin B (18), but the tip of the pipette was filled with ionophore-free solution to allow formation of the seal. The access resistance was reduced to 10–30 M $\Omega$  in less than 15 min, and the process was slightly faster with amphotericin B than with nystatin. The reference electrode was placed in a KCl solution linked to the bath with an Agar bridge; the resulting liquid junction potential was smaller than 2 mV and has been neglected. The cell was voltage clamped (Axopatch 1D, Axon Instruments, Foster City, CA) at a holding potential of  $-90$  mV and depolarized as indicated. The currents were filtered at 1–2 kHz and sampled at 6.2 kHz. Leak was subtracted either digitally after the experiment or automatically by a P/4 protocol (pclamp 6, Axon Instruments).

### $[\text{Ca}^{2+}]_i$ measurements

$[\text{Ca}^{2+}]_i$  was determined either with fura-2 in populations of cells freshly isolated and purified on a Percoll density gradient, as previously described in detail (13), or in single voltage-clamped cells loaded with the probe fluo-3 in combination with the patch-clamp technique in the perforated patch configuration. For this purpose, bovine glomerulosa cells were plated on small glass coverslips coated with Cell-Tak and cultured for 2–4 days. The cells were then incubated for 45 min at 37°C in the presence of 8  $\mu\text{M}$  fluo-3 acetoxymethyl ester and 6.25% (wt/vol)

pluronic acid, washed in bath solution, and immediately mounted in a home-made patch-clamp recording chamber maintained in the dark. Fluo-3 fluorescence (excitation at 470 nm and emission at 540 nm) was monitored on a Zeiss Axiovert 10 inverted microscope (Zeiss, New York, NY) equipped with a Bio-Rad CRS-400 microfluorimeter (Bio-Rad, Glattbrugg, Switzerland). The fluorescent signal was sampled at 2 Hz and recorded using the CRS-400 software (Bio-Rad).

### Determination of aldosterone formation

Measurement of aldosterone production from freshly prepared or cultured glomerulosa cells was performed as described previously (8). Glomerulosa cells, when cultured for 3 days, were incubated at 37°C in multiwell plates containing a modified Krebs-Ringer medium and various concentrations of potassium and pharmacological inhibitors of  $\text{Ca}^{2+}$  channels. At the end of the incubation period, the aldosterone content of the medium was determined by direct RIA, using a commercially available kit (Diagnostic Systems Laboratories, Webster, TX).

### Statistical analysis

When appropriate, statistical significance of differences was determined by paired Student's *t* test and corrected according to Bonferroni's method. A difference was considered statistically significant at a corrected  $P < 0.05$ .

### Results

#### Contribution of T- and L-type $\text{Ca}^{2+}$ channels to the depolarization-evoked cytosolic $\text{Ca}^{2+}$ signal

To determine the role of T-type  $\text{Ca}^{2+}$  channels in the sustained  $[\text{Ca}^{2+}]_i$  response to cell depolarization induced in bovine adrenal glomerulosa cells during stimulation by extracellular  $\text{K}^+$  or AngII,  $\text{Ca}^{2+}$  currents and  $[\text{Ca}^{2+}]_i$  variations were recorded in the same cell by combining the patch-clamp technique, in its perforated patch configuration, and the microfluorimetry of fluo-3. Cell depolarization to 0 mV, from a holding potential of  $-100$  mV, induced a marked increase in  $[\text{Ca}^{2+}]_i$ , which rapidly returned to basal values upon cell repolarization, demonstrating the ability of the  $\text{Ca}^{2+}$  pumps to maintain a low  $[\text{Ca}^{2+}]_i$  in the absence of stimulation (Fig. 1A). When the cell was gradually depolarized in a stepwise manner, no response was observed below  $-60$  mV;  $[\text{Ca}^{2+}]_i$  then rapidly rose to reach a maximum at  $-30$  mV and slightly decreased at more positive potentials (Fig. 1, A and D). Once again,  $[\text{Ca}^{2+}]_i$  returned to basal levels immediately after cell repolarization, and a novel response could be evoked by a large depolarization to 0 mV. The steady state current through T-type channels was also determined in the same cell, as described previously (13), by measuring slowly deactivating  $\text{Ca}^{2+}$  currents evoked upon repolarization at  $-65$  mV (not shown). Analysis of tail currents allowed us to establish the activation and inactivation curves of T channels (Fig. 1C); the expected relative steady state current through these channels was calculated from Ohm's law and compared to the depolarization-evoked  $[\text{Ca}^{2+}]_i$  response (Fig. 1D). A clear dissociation was observed between the sustained  $\text{Ca}^{2+}$  influx expected to occur through T channels ( $I_{\text{ss}}$ ) and the measured  $[\text{Ca}^{2+}]_i$  response (fluo-3 signal). Because this dissociation appeared much more pronounced at more positive voltages, a large participation of L-type channels to  $\text{Ca}^{2+}$  influx was hypothesized.

To reduce as much as possible the contribution of L-type channels, the cell was then exposed to highly specific inhibitors of L-type channels. As shown in Fig. 1B, the addition of

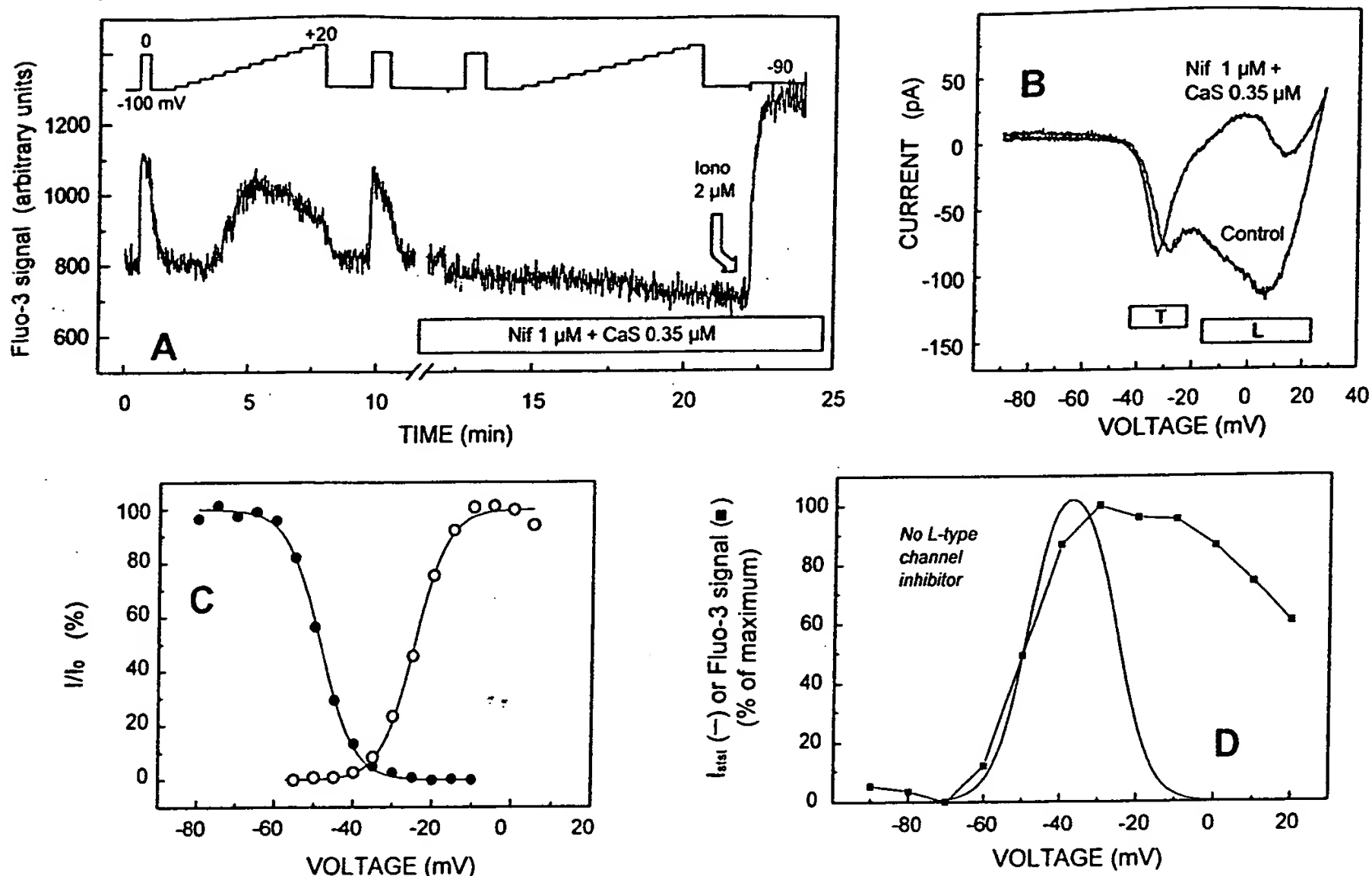
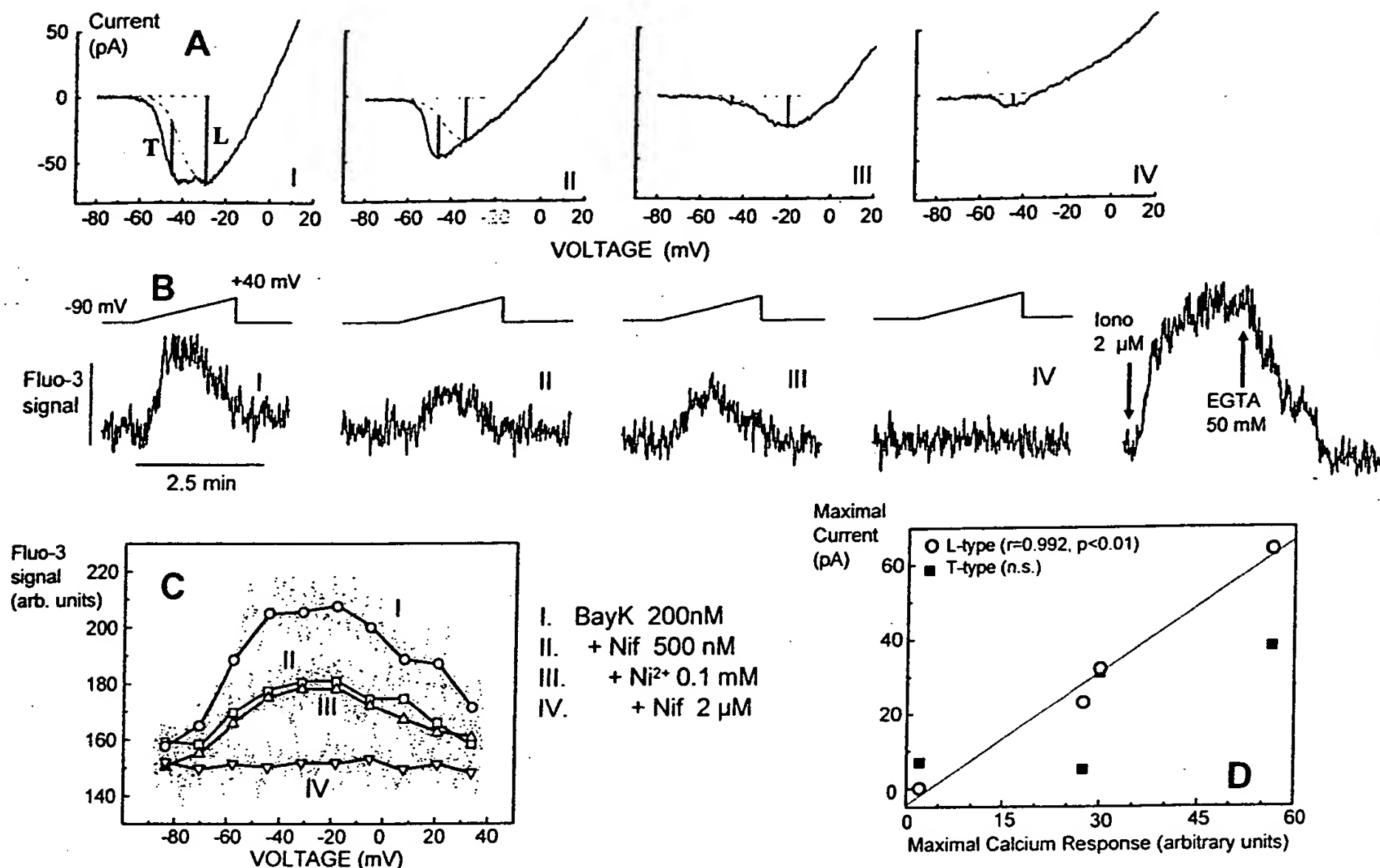


FIG. 1. Minor role of T-type calcium channels in depolarization-evoked cytosolic calcium response. A, Voltage-dependent cytosolic  $\text{Ca}^{2+}$  signal. A single fluo-3-loaded bovine adrenal glomerulosa cell was voltage clamped in the nystatin perforated patch configuration of the patch-clamp technique, as described in detail in *Materials and Methods*. Cytosolic  $\text{Ca}^{2+}$ -dependent fluorescence was recorded at a holding potential of  $-100$  mV and upon various step depolarizations (the voltage protocol is indicated on the top of the trace). After 12 min of recording, nifedipine ( $1 \mu\text{M}$ ) and calci-septine ( $0.35 \mu\text{M}$ ) were added together to the medium, the protocol was repeated, and  $2 \mu\text{M}$  ionomycin was introduced at the end of the experiment to induce a maximal  $\text{Ca}^{2+}$  response. B, Selectivity of nifedipine and calci-septine for L-type channels. T- and L-type  $\text{Ca}^{2+}$  currents were evoked in the same cell as in A by ramp depolarization from  $-110$  to  $+40$  mV (duration, 1.8 sec), before the fluorescence recording (Control), and immediately after addition of Nif and CaS to the bath (during the gap of the time scale in A). Currents are expressed as a function of the voltage value reached during the ramp protocol (33). C, Activation and inactivation curves of T-type channels. Voltage-dependent activation (O) and inactivation ( $\bullet$ ) of slowly deactivating currents were measured in this same cell at the beginning of the experiment as described previously (13). Current amplitudes were plotted as a function of test voltage, fitted to Boltzman's equation, and normalized to the maximal current ( $I_0$ ).  $V_{1/2}$  values (the voltage corresponding to half the maximal effect) were  $-24.7$  and  $-48.1$  mV for activation and inactivation, respectively, in this particular cell. D, Comparison of steady state current through T channels and membrane potential-dependent  $[\text{Ca}^{2+}]_c$  concentration. The theoretical steady state current ( $I_{ss}$ ) was calculated from the activation and inactivation curves according to Ohm's equation as previously described (13) and expressed as a percentage of the maximal current as a function of voltage (smooth curve). The fluo-3 signal observed during the first gradual stepwise depolarization (A) was averaged for each potential value and normalized ( $\blacksquare$ ). Similar results were obtained in each of five separate cells in which currents were recorded in combination with fluo-3 signal.

$1 \mu\text{M}$  nifedipine and  $0.35 \mu\text{M}$  calci-septine specifically decreased L current evoked by a ramp depolarization, but did not affect the lower threshold, transiently activated, T-type current. Surprisingly, the disappearance of L current was accompanied by complete abolition of the  $[\text{Ca}^{2+}]_c$  response to cell depolarization, although a large entry of  $\text{Ca}^{2+}$ , induced by the  $\text{Ca}^{2+}$  ionophore ionomycin, was still detectable with fluo-3 (Fig. 1A).

These results, therefore, suggested that T channels play only a minor role in the elevation of  $[\text{Ca}^{2+}]_c$  observed upon glomerulosa cell depolarization. This hypothesis was reinforced by the observation of a significant correlation of the maximal  $[\text{Ca}^{2+}]_c$  increase elicited during a slow ramp depolarization (duration, 2 min), under various pharmacological conditions (Fig. 2B), with the maximal amplitude of L cur-

rents, but not with that of T currents, induced by a similar, but faster, depolarization (duration, 1.8 sec; Fig. 2A). Indeed, the amplitudes of T- and L-type currents could be independently manipulated in the same cell by adding consecutively to the bath: 1)  $200 \text{ nM}$  BayK 8644, an agonist of L-type channels; 2)  $500 \text{ nM}$  nifedipine, an antagonist of L-type channels; 3)  $0.1 \text{ mM}$   $\text{Ni}^{2+}$ , an inorganic blocker of T-type channels; and finally, 4)  $2 \mu\text{M}$  nifedipine. In the presence of BayK 8644 alone, L current was predominant in this cell, and the maximal current amplitude was slightly shifted toward more negative potentials, partially overlapping T-type current (Fig. 2A-I). Analysis of the fluo-3 signal in response to cell depolarization (Fig. 2, B-I and C-I) showed that  $[\text{Ca}^{2+}]_c$  was maximal between  $-45$  and  $-15$  mV and decreased thereafter. The addition of  $500 \text{ nM}$  Nif did not completely block



**FIG. 2.** Correlation between L-type calcium channel activity and depolarization-evoked cytosolic calcium response. Calcium currents (A) and fluo-3 signals (B) in response to a ramp depolarization were consecutively recorded in a single voltage-clamped glomerulosa cell maintained in the amphotericin B perforated patch configuration and in the presence of various pharmacological modulators of T- and L-type channels. Phase I, BayK (200 nM); phase II, BayK plus nifedipine (500 nM); phase III, BayK, Nif, and  $\text{Ni}^{2+}$  (0.1 mM); phase IV, BayK,  $\text{Ni}^{2+}$ , and Nif (2.5  $\mu$ M). Calcium currents (A) were evoked at the beginning and end of each phase by cell depolarization from  $-110$  to  $+40$  mV (duration, 1.8 sec) and were averaged and expressed as a function of the instantaneous voltage, as described in Fig. 1B. The maximal amplitudes of T- and L-type currents were estimated to occur at  $-45$  mV and approximately  $-30$  mV, respectively. Calcium responses were recorded during the 2-min depolarization protocol indicated at the top of the traces (B), and ionomycin (2  $\mu$ M) followed by EGTA (50 mM) were added at the end of the experiment to determine, respectively, the maximal and minimal signals of fluo-3. C, Fluo-3 signal during each phase was smoothed by averaging every 25 consecutive data points (the sampling rate during data acquisition was 2 Hz, corresponding to a point every 0.54 mV), and the results were expressed as a function of the mean voltage of the averaging domain. D, Correlation between L-type current and  $[\text{Ca}^{2+}]_c$ . The maximal T- and L-type currents estimated in A were compared to the maximal fluo-3 signal determined in C, and the correlation was assessed by least square linear regression analysis.

L-type channels, presumably because of the continuous presence of BayK, but reduced the maximal amplitude of L current by approximately 50% without significantly affecting that of T-type current (Fig. 2A-II). Concomitantly, fluo-3 signal amplitude was reduced by about half, without any apparent shift in its maximum (Fig. 2, B-II and C-II). In contrast,  $\text{Ni}^{2+}$  almost completely abolished T-type current (Fig. 2A-III), without modifying either L current or  $[\text{Ca}^{2+}]_c$  (Fig. 2, B-III and C-III). Finally, complete inhibition of L-type channels with an excess of Nif resulted in complete inhibition of the  $[\text{Ca}^{2+}]_c$  response to cell depolarization (Fig. 2, A-IV, B-IV, and C-IV), although the addition of a  $\text{Ca}^{2+}$  ionophore (ionomycin) and a  $\text{Ca}^{2+}$  chelator (EGTA) resulted in the expected variations in  $[\text{Ca}^{2+}]_c$ . When the maximal  $\text{Ca}^{2+}$  current amplitudes through each channel were compared to the maximal  $[\text{Ca}^{2+}]_c$  responses (Fig. 2D), a significant correlation was observed only with L-type currents, suggesting that the ac-

tivation of L-type channels is principally responsible for the voltage-dependent variations of  $[\text{Ca}^{2+}]_c$ .

To demonstrate that L-type channels are involved in the  $[\text{Ca}^{2+}]_c$  response to even low, physiological concentrations of extracellular potassium, fura-2-loaded cells in suspension were exposed to various pharmacological agents before being gradually depolarized by consecutive additions of KCl to the medium. Figure 3A shows that 100 nM BayK 8644, which by itself had only a minor effect on basal  $[\text{Ca}^{2+}]_c$ , markedly potentiated the response to KCl addition. When the mean  $[\text{Ca}^{2+}]_c$  increase was determined and expressed as a function of the actual  $\text{K}^+$  concentration in the medium (Fig. 3B), BayK 8644 (100 nM) appeared to considerably increase and Nif (50 nM) to reduce the  $[\text{Ca}^{2+}]_c$  response at each KCl concentration tested, without shifting the maximum. To determine whether T-type channels could be primarily responsible for the  $[\text{Ca}^{2+}]_c$  response to low  $\text{K}^+$  concentrations, whereas L-type



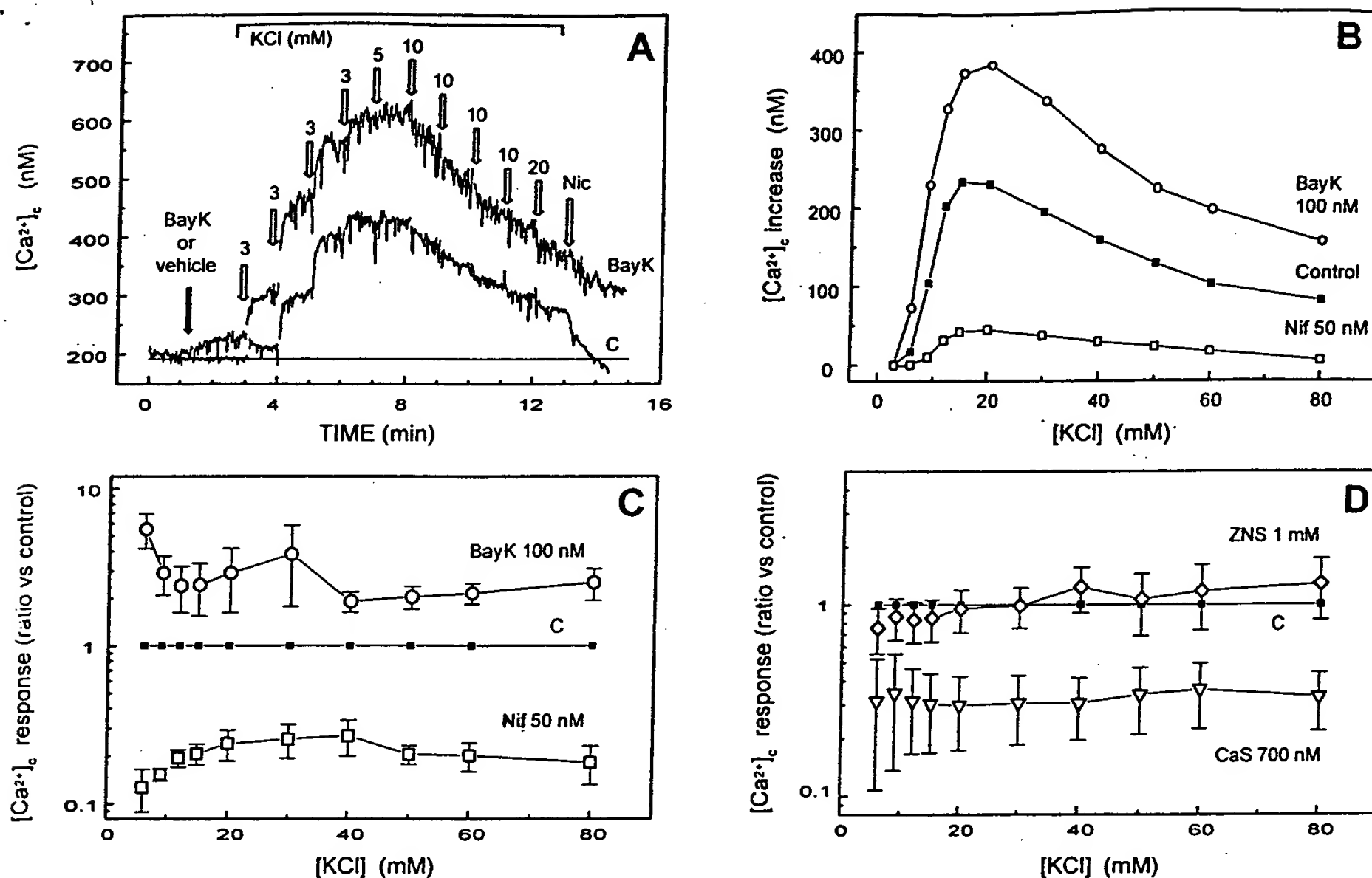


FIG. 3. Involvement of L-type channels in the cytosolic calcium response to physiological concentrations of potassium. A, Effect of BayK on the  $[Ca^{2+}]_c$  response to extracellular  $K^+$ . Freshly prepared glomerulosa cells were loaded with fura-2, as described in *Materials and Methods*, and exposed to increasing concentrations of extracellular  $K^+$  (basal  $K^+$ , 3 mM; arrows indicate the time and numbers the millimolar increases in  $K^+$ ). One batch of cells was previously exposed (black arrow) to 100 nM BayK, whereas the control cells received dimethylsulfoxide only (C). Both groups were exposed to 2  $\mu$ M nicardipine at the end of the experiment. The dotted line represents basal  $[Ca^{2+}]_c$ . B, Modulation of the  $K^+$ -dependent  $[Ca^{2+}]_c$  signal by BayK and nifedipine. The same protocol as that described in A was performed with cells exposed to 50 nM nifedipine, 100 nM BayK, or dimethylsulfoxide, and the mean increase in  $[Ca^{2+}]_c$  over basal was expressed as a function of the  $K^+$  concentration. C and D, Efficacy of various  $Ca^{2+}$  channel modulators as a function of the extracellular  $K^+$  concentration. At each extracellular  $K^+$  concentration of the protocol described in A, the ratio between the  $[Ca^{2+}]_c$  response measured in the presence and the absence of 100 nM BayK, 50 nM Nif, 1 mM zonisamide (ZNS), or 700 nM calciseptine (CaS) was calculated. Results are the mean  $\pm$  SEM from four to six experiments performed in duplicate.

channels would be only involved at higher, supraphysiological  $K^+$  concentrations, the sensitivity of the response to dihydropyridines was analyzed as a function of the KCl concentration. For this purpose, the ratio of the response after treatment with each dihydropyridine over the response in control untreated cells was calculated (Fig. 3C). The cells did not appear to be less sensitive at lower  $K^+$  concentrations, as would be the case if  $Ca^{2+}$  influx occurred through T-type channels under these conditions. Similarly, when L-type channels were specifically inhibited with 700 nM calciseptine (Fig. 3D), the  $[Ca^{2+}]_c$  response was inhibited in the same proportion over the complete range of KCl concentrations tested. This demonstrates that the  $K^+$ -induced  $Ca^{2+}$  signal is mediated by a single, pharmacologically homogeneous class of channel, highly sensitive to dihydropyridines and calciseptine. In contrast, treatment of the cells with 1 mM zonisamide, an antiepileptic agent with a low selectivity for T-type channels (19), had almost no effect on the  $[Ca^{2+}]_c$  response (Fig. 3D).

It, therefore, appears that the cytosolic  $Ca^{2+}$  response to extracellular  $K^+$  measured in bovine adrenal glomerulosa cells with fluorescent probes is principally, if not entirely, due to activation of dihydropyridine-sensitive L-type  $Ca^{2+}$  channels even at lower  $K^+$  concentrations when the cells are only slightly depolarized. In contrast, the modulation of  $Ca^{2+}$  influx through T-type channels is not detectable in the cytosol (see *Discussion*).

#### *Lack of correlation between cytosolic calcium and aldosterone secretion*

The relationship between the levels of  $[Ca^{2+}]_c$  maintained by  $K^+$  and the activation of aldosterone secretion by the same agonist was investigated by modulating  $Ca^{2+}$  channel activity with nifedipine, BayK 8644, or zonisamide (Fig. 4). Increasing concentrations of Nif, between 1 nM and 3  $\mu$ M (Fig. 4, left panels), markedly and monotonously reduced  $[Ca^{2+}]_c$  stimulated by 12 mM  $K^+$ , even below resting levels, indicat-

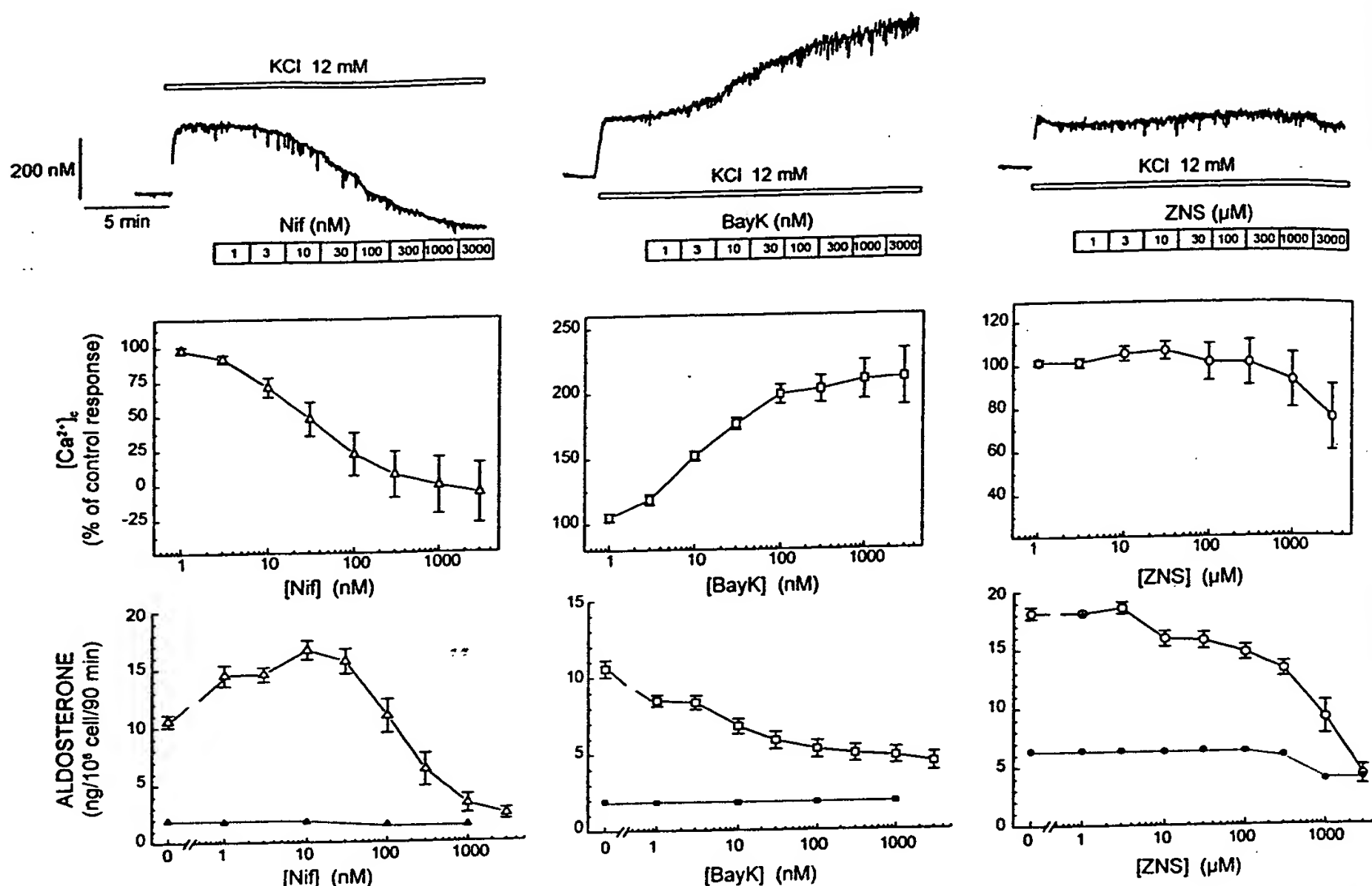


FIG. 4. Lack of correlation between the  $[Ca^{2+}]_i$  and aldosterone responses to potassium. The effects of increasing concentrations of nifedipine, BayK 8644, and zonisamide on  $K^+$ -induced  $[Ca^{2+}]_i$  and steroidogenic responses were examined in freshly prepared bovine glomerulosa cells. Representative traces of  $[Ca^{2+}]_i$  measured in cell populations with the fura-2 technique are shown in the *top panels*. Potassium and drug concentrations in the medium are indicated at the *bottom* or the *top* of each trace. Basal  $[Ca^{2+}]_i$  (measured in a Krebs-Ringer medium containing 3 mM  $K^+$ ) was  $207 \pm 5$  nM (mean  $\pm$  SEM;  $n = 9$ ). The effect of increasing concentrations of each drug on  $[Ca^{2+}]_i$  stimulated by a 9-mM increase in  $K^+$  was expressed as a percentage of the response to KCl in the absence of the drug (*center panels*). Aldosterone secretion in KCl was determined and expressed as a percentage of the response to KCl in the absence of the drug (*bottom panels*) after 90-min incubation of the cells in the presence of increasing concentrations of Nif, BayK, ZNS, and either low (3 mM; *closed symbols*) or high  $K^+$  (12 mM; *open symbols*). Results are the mean  $\pm$  SEM from three to seven experiments, and aldosterone was measured in duplicate.

ing the presence of a small basal  $Ca^{2+}$  influx sensitive to dihydropyridines, as previously described in these cells (8). The effect of Nif on aldosterone secretion followed a different pattern. While concentrations of Nif above 100 nM efficiently inhibited  $K^+$ -induced steroidogenesis, aldosterone output was significantly potentiated by the presence of the  $Ca^{2+}$  antagonist at concentrations between 1–30 nM. Basal aldosterone production was not affected by Nif.

As expected, the agonist BayK 8644 potentiated the  $[Ca^{2+}]_i$  response to 12 mM  $K^+$ , increasing the signal by approximately 110% at the maximal concentration; however, the same agent markedly reduced, in a concentration-dependent manner, aldosterone secretion sustained by high  $K^+$  (12 mM) without affecting basal steroidogenesis (Fig. 4, *middle panels*).

Finally, zonisamide (*right panels*), the slightly selective inhibitor of T-type channels, only minimally affected  $K^+$ -induced  $[Ca^{2+}]_i$  compared to Nif or BayK 8644, but markedly decreased both stimulated and basal aldosterone secretion.

By using various pharmacological agents acting selectively on T- or L-type channels, it was, therefore, possible to illustrate the dissociation existing between the levels of bulk

$[Ca^{2+}]_i$  as measured with classical fluorescent probes, and aldosterone synthesis upon stimulation with high  $K^+$  concentrations.

#### Correlation between T-type channel activity and potassium-stimulated aldosterone production

To determine the specificity of the  $Ca^{2+}$  channel-modulating agents employed in this study, it was necessary to examine the effects of various concentrations of these agents on T- and L-type channels. The *upper panels* of Fig. 5 show the pattern of  $Ba^{2+}$  currents recorded in the whole cell configuration of the patch-clamp technique and elicited by a gradual depolarization of the cells, as described in Figs. 1B and 2A. The size of the inward current with the lower threshold, peaking at about  $-30$  mV, was systematically compared to the amplitude of the slowly deactivating current induced in the same cell (not shown), measured as described in detail previously (13). Both methods for isolating and measuring T-type channel activity were closely correlated ( $r = 0.928$ ;  $n = 48$ ;  $P < 0.001$ ), demonstrating that the voltage ramp protocol

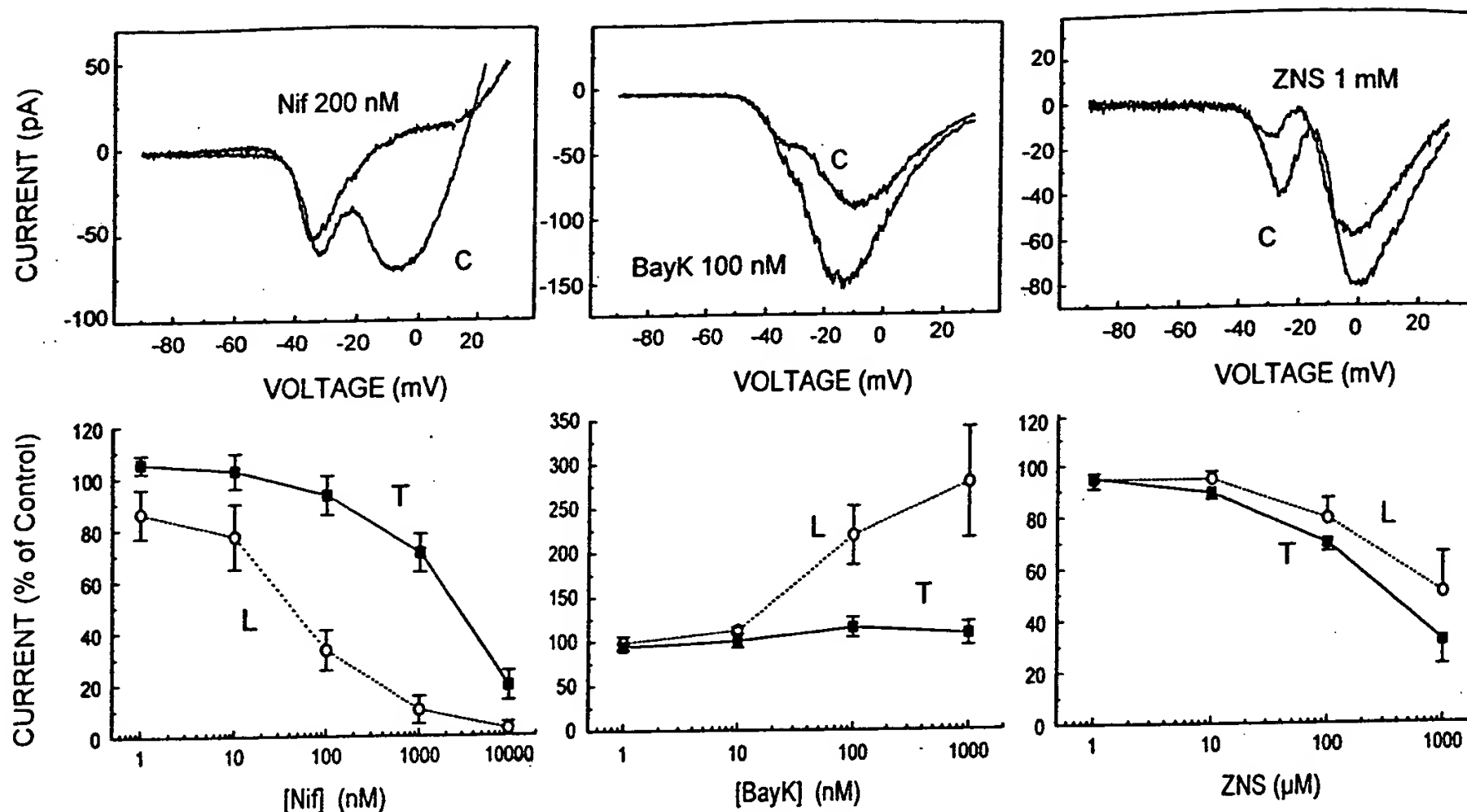


FIG. 5. Selectivity of nifedipine, BayK 8644, and zonisamide for T- and L-type calcium channels. T- and L-type  $\text{Ba}^{2+}$  currents were partially resolved by ramp depolarizations (from  $-110$  up to  $+40$  mV during 1.8 sec) in the whole cell configuration of the patch-clamp method. An example of the effect of 200 nM Nif, 100 nM BayK 8644, and 1 mM ZNS on  $\text{Ba}^{2+}$  currents is presented in the *upper panels* (C, control trace, recorded before drug addition). Concentration-dependent curves of inhibition or activation of each current by Nif, BayK, and ZNS were established by expressing the value of the specific peak current, measured after drug addition, as a percentage of the control current (*lower panels*). Data are the mean results obtained from 5–14 independent cells.

TABLE 1. Correlation between potassium-induced aldosterone secretion and T channel activity

Drug	Activity (% of control)			
	Aldosterone	T Channel	L Channel	Cytosolic $\text{Ca}^{2+}$
Nif (100 nM)	$69.5 \pm 13.4$ (9)	$93.2 \pm 7.5$ (7)	$33.3 \pm 7.8$ (5)	$21.5 \pm 5.4$ (8)
Nic (10 nM)	$124.6 \pm 13.5$ (13)	$131.4 \pm 23.2$ (5)	$58.9 \pm 13.2$ (5)	$26.8 \pm 5.5$ (5)
BayK (100 nM)	$71.3 \pm 7.3$ (9)	$115.5 \pm 11.3$ (10)	$219.1 \pm 33.3$ (7)	$224.6 \pm 43.9$ (7)
ZNS (100 $\mu\text{M}$ )	$87.3 \pm 4.2$ (10)	$75.9 \pm 3.8$ (14)	$79.2 \pm 7.9$ (7)	$101.5 \pm 8.5$ (7)
TET (10 $\mu\text{M}$ )	$58.8 \pm 5.1$ (11)	$51.9 \pm 9.0$ (13)	ND	ND

Aldosterone secretion from freshly prepared or cultured glomerulosa cells stimulated with 12 mM  $\text{K}^+$  was determined in control (untreated) cells and in cells exposed to drugs at a concentration (100 nM nifedipine, 10 nM nicardipine, 100 nM BayK 8644, 100  $\mu\text{M}$  zonisamide, or 10  $\mu\text{M}$  tetrandrine) yielding optimal selectivity for L- or T-type channels. The effect of each drug on aldosterone production, expressed as a percentage  $\pm$  SEM of the control value, is compared to the effect of the same drug concentration on T and L channel activity, assessed as described in Fig. 5, and on  $[\text{Ca}^{2+}]_i$ , determined with fura-2. Least square linear regression analysis revealed a positive correlation between aldosterone secretion and T-type channel activity ( $r = 0.97$ ). No correlation was observed between any other groups. The values in parentheses indicate the number of independent determinations. ND, Not determined.

allows discrimination between T- and L-type currents. The ratio between L- and T-type currents under control conditions varied greatly from one cell to the other [see, for example, the control (C) traces in the *upper panels* of Fig. 5], and the effects of the drugs were, therefore, expressed as the amplitude of each current as a percentage of the corresponding control current measured before treatment (Fig. 5, *lower panels*).

Nifedipine inhibited both currents, but T-type channels were affected by approximately 100 times higher concentrations of the drug, showing that it is possible to substantially reduce L-type channel activity without markedly affecting

T-type channels at concentrations of Nif ranging from 100–1000 nM.

The effect of BayK 8644 on T- and L-type channels was even more contrasted, as this compound, at concentrations above 10 nM, considerably increased L-type current by up to 170%, whereas the current flowing through T-type channels was not significantly affected.

Finally, zonisamide was slightly more efficient in inhibiting T-type current compared to L-type current, although millimolar concentrations of this agent also markedly affected the latter.

The actions of these drugs on T- and L-type channel ac-

tivity as well as on  $[Ca^{2+}]_c$  were then compared to their abilities to prevent aldosterone secretion upon stimulation with 12 mM  $K^+$  (Table 1). Tetrandrine, an alkaloid relatively selective for T-type channels (11), and nicardipine, a dihydropyridine efficiently blocking both T- and L-type channels when used at micromolar concentrations (8), were also included in this comparison. The effect of each drug on potassium-induced steroidogenesis, when employed at a concentration providing an optimal selectivity for one type of  $Ca^{2+}$  channel, appeared to correlate much better with their actions on T channel ( $r = 0.69$ ) than on L channel activity ( $r = -0.37$ ). In contrast,  $[Ca^{2+}]_c$  measured in the presence of the same drugs was more closely related to L-type currents ( $r = 0.97$ ) than to T-type currents ( $r = 0.03$ ), suggesting distinct cellular functions for these channels.

*Phorbol ester inhibits  $Ca^{2+}$  influx through T-type channels as well as steroidogenesis without affecting  $[Ca^{2+}]_c$  concentration*

An additional illustration of the discrepancy between  $[Ca^{2+}]_c$  on the one hand, and aldosterone synthesis and T channel activity, on the other hand, is provided by the action

of the phorbol ester, PMA. Through the direct activation of protein kinase C (PKC), PMA mimics some of the effects of AngII, among which is an inhibition of T-type channel activity (13). This inhibition is attributable to a significant shift ( $P < 0.01$ ) in the activation curve of the channel by approximately 7 mV (at half-activation) toward more positive voltage values (Fig. 6A). In contrast, the inactivation of the channel is not affected by this treatment. As a consequence, the size of the permissive window of voltage is reduced, resulting in a substantial decrease in the expected steady state current through these channels (Fig. 6B). The reduction of  $Ca^{2+}$  influx resulting from T channel inhibition by PMA was not reflected by the levels of  $[Ca^{2+}]_c$  as shown in Fig. 6C. In this experiment, fura-2-loaded cells were first stimulated with 9 mM KCl to maximally activate T- and L-type channels, and when the  $[Ca^{2+}]_c$  response had reached a plateau, 500 nM PMA was added. No effect of PMA was observed, suggesting that this compound does not significantly affect L-type channels. However, upon stimulation with AngII, after a transient  $Ca^{2+}$  release phase mediated by inositol 1,4,5-trisphosphate, a marked reduction of  $[Ca^{2+}]_c$  was induced, as previously described (13), possibly because L-type channels are con-

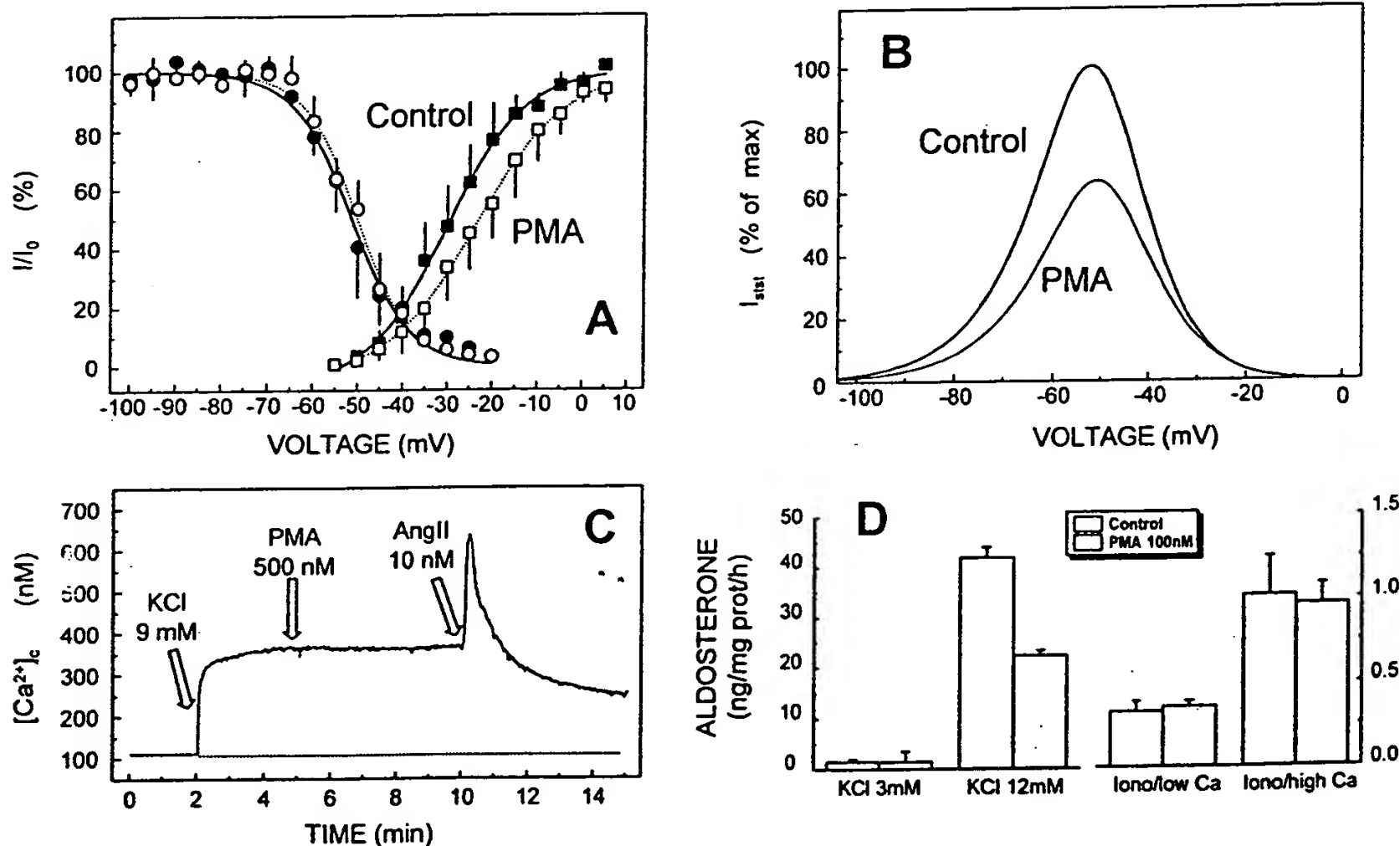


FIG. 6. Inhibition by PMA of T-type channel activity and potassium-stimulated aldosterone secretion, but not of the  $[Ca^{2+}]_c$  response. A, PMA effect on the T channel activation curve. Glomerulosa cells were voltage clamped in the whole cell configuration, and  $Ba^{2+}$  currents were elicited as described in Fig. 1C, either before (Control) or 3 min after the addition of 500 nM PMA. Tail currents of T channels were analyzed for each cell separately; data were fitted to Boltzman's equation and normalized to  $I_0$  (maximal current) before being averaged. Activation (■ and □) and inactivation (● and ○) curves were established, as described in Fig. 1, before (■ and ●) and after (□ and ○) exposure to PMA (mean  $\pm$  SEM;  $n = 4$  cells). The mean  $V_{1/2}$  for channel inactivation was  $-51.4$  mV before and  $-50.1$  mV after PMA application, whereas the  $V_{1/2}$  for activation was shifted from  $-30.2$  to  $-23.6$  mV by the same treatment. B, Inhibition of the steady state current by PMA. The theoretical steady state current was calculated from Ohm's function, as described in Fig. 1D, before and after the addition of PMA. C, Lack of effect of PMA on  $[Ca^{2+}]_c$ . Fura-2-loaded cells were consecutively exposed to 9 mM KCl, 500 nM PMA, and 10 nM AngII, and  $[Ca^{2+}]_c$  was monitored as described in *Materials and Methods*. The trace is representative of at least 10 independent observations from various cell preparations that gave similar results. D, Effect of PMA on calcium-induced aldosterone secretion. Aldosterone secretion was stimulated in the presence or absence of 100 nM PMA, either in intact cultured glomerulosa cells challenged with 12 mM  $K^+$  (left ordinate) or in freshly prepared, ionomycin (1  $\mu$ M)-treated cells (right ordinate) by increasing their  $[Ca^{2+}]_c$  from 50 (low Ca) to 600 nM (high Ca).



controlled by AngII through a completely distinct mechanism, for example involving G proteins.

Despite the lack of effect of PMA on  $[Ca^{2+}]_c$ , KCl-stimulated aldosterone secretion was strongly inhibited (46%) by this agent (Fig. 6D), whereas basal aldosterone (at 3 mM  $K^+$ ) was not affected. This inhibition did not appear to result from a direct action of PKC on the steroidogenic pathway, but, rather, from a reduction of  $Ca^{2+}$  influx into the cell, because when  $Ca^{2+}$  channels were bypassed with a  $Ca^{2+}$  ionophore such as ionomycin, the stimulation of steroidogenesis induced by increasing extracellular  $Ca^{2+}$  was insensitive to PMA (Fig. 6D).

### Discussion

This study has allowed us to demonstrate that KCl-induced variations in  $[Ca^{2+}]_c$  and aldosterone secretion in bovine adrenal glomerulosa cells can be dissociated with pharmacological agents possessing some specificity for T-type or L-type  $Ca^{2+}$  channels. Indeed, whereas  $[Ca^{2+}]_c$  appeared to essentially reflect L-type channel activity, the steroidogenic response was closely related to T-type channels.

A sustained influx of  $Ca^{2+}$  through T-type channels upon physiological depolarization of the cell by extracellular  $K^+$  is theoretically possible because of the overlap of the activation and steady state inactivation curves of the channel (12, 13). However, because of the low percentage (<1%) of T channels statistically open during a prolonged stimulation with  $K^+$ , this current is expected to be very small. A raw estimation of the amplitude of this current by noise analysis revealed a sustained, nifedipine-resistant (500 nM) and  $Ni^{2+}$ -sensitive current of approximately 0.1 pA at -40 mV (data not shown). This very low amplitude of the steady state current through T-type channels certainly explains the lack of contribution of these channels to the cytosolic response, which is mainly maintained by the strong (at least 100-fold larger)  $Ca^{2+}$  current through L-type channels, but strikingly contrasts with its crucial role in the regulation of steroidogenesis.

One possible explanation for this paradox is the existence of a very efficient coupling between T-type channels and the  $Ca^{2+}$  target sites involved in the regulation of aldosterone formation. It is now well recognized that the mitochondria play a crucial role in this regulation (3), not only because these organelles contain the steroidogenic enzymes responsible for the early, rate-limiting, and late steps of aldosterone synthesis, but also because it has been shown that the stimulatory action of  $Ca^{2+}$  requires its entry into the mitochondrial matrix through the ruthenium red-sensitive uniporter (20). A close apposition of the mitochondria and of T-type channels or the existence of a sort of  $Ca^{2+}$  pipeline, possibly involving the endoplasmic reticulum and transporting  $Ca^{2+}$  from the mouth of the channel to the proximity of the mitochondrion with a very restricted passage of  $Ca^{2+}$  through the cytosol, could provide a functional link between  $Ca^{2+}$  influx through T-type channels and the activation of steroidogenesis.

In favor of this model is the observation that in some tissues, basal and stimulated intramitochondrial  $[Ca^{2+}]_m$ , as assessed by targeted aequorin (21), are relatively high compared to the concentrations present in the bulk cytosol (22).

Although this gradient of  $[Ca^{2+}]$  between the mitochondrial matrix and the cytosol could be partially due to the very negative potential of the inner mitochondrial membrane (23), the presence of mitochondria in some cellular microdomains with high  $[Ca^{2+}]_c$  has been also demonstrated (22).

It, therefore, appears that T- and L-type channels might play distinct functional roles in glomerulosa cells. T-type channels are designed to conduct low amounts of  $Ca^{2+}$  directly to the mitochondria, where the cation can affect key steps of steroidogenesis, such as cholesterol transport to the inner membrane (24), whereas L-type channels are responsible for the entry of much larger amounts of  $Ca^{2+}$ , which spreads throughout the cytosol. The role of this cytosolic  $Ca^{2+}$  has not yet been clearly defined and could relate to more general cell functions, such as gene expression or cytoskeleton remodeling. However, an excess of cytosolic  $Ca^{2+}$  exerts a negative effect on aldosterone biosynthesis (15, 24), and this could explain the observed potentiation of the steroidogenic action of  $K^+$  by low, L-type channel-specific concentrations of nifedipine as well as its inhibition by BayK 8644, as observed by others (15, 16, 25). Indeed, the inhibition of aldosterone by BayK 8644 was observed only at  $K^+$  concentrations greater than 10 mM (not shown) and could, therefore, be only partially attributed to its known inhibitory action on T-type channels (26, 27).

A specific role for various types of channels has been clearly demonstrated in the central nervous system, where neurotransmitter release is attributed to presynaptic N- and P-type  $Ca^{2+}$  channel activation and is not affected by inhibition of L-type channels (28). This selectivity has been postulated to result from a privileged localization of some channels in the membrane, permitting a more efficient supply of  $Ca^{2+}$  to the secretory apparatus. This hypothesis has been recently reinforced by the observation that the N-type channel strongly binds, in a  $Ca^{2+}$ -dependent fashion, to syntaxin, a membrane protein allowing docking and fusion of synaptic vesicles during neurosecretion (29). Interestingly, syntaxin is able to modulate by itself the activity of N-type channels (30), suggesting considerable cross-talk between N-type channels and the synaptic core complex. The possibility of a similar interaction between glomerulosa cell T-type channels and some cellular proteins clearly requires further investigation.

What could be the advantage for glomerulosa cells of using specific  $Ca^{2+}$  pathways for triggering steroidogenesis? Firstly, because the main site of  $Ca^{2+}$  action is localized in the mitochondria, a small amount of  $Ca^{2+}$  entering the cell should be sufficient during activation, but this  $Ca^{2+}$  needs to reach its target with a minimal dispersion in the cytosol. Secondly, it is now widely admitted that the domain of  $Ca^{2+}$  action in the cytosol is restricted to very limited areas (31), its diffusion being prevented by its binding to slowly mobile or immobile buffers. Depending upon the distribution of the mitochondria within the cell, the presence of a  $Ca^{2+}$  pipeline might, therefore, be an absolute requirement. Finally, in contrast to L-type channels, T-type channels are not subject to down-regulation by  $Ca^{2+}$  (possibly because they are preserved during their relatively longer inactivation phase) and, therefore, can maintain a sustained stimulation of steroidogenesis during chronic elevation of extracellular potassium.

The inhibition of T channel activity and aldosterone se-

cretion observed upon treatment with phorbol ester is presumably due to activation of PKC. This action of PKC appears to be restricted to T-type channels, because  $[Ca^{2+}]_i$  is not affected. However, a definitive proof would require a complete evaluation of PMA action on L-type channels. Interestingly, AngII, when added after PMA, markedly reduced  $[Ca^{2+}]_i$ , suggesting a PKC-independent regulation of L-type channels by the hormone, possibly through a GTP-binding protein. This confirms our previous observation that in adrenal glomerulosa cells, AngII action involves stimulatory as well as inhibitory pathways (13, 32, 34).

In conclusion, the demonstration that various  $Ca^{2+}$  channels may play distinct roles in the glomerulosa cell stresses the need for the development of more specific drugs to efficiently and selectively control individual channel functions. This approach should be particularly relevant in the treatment of hypertension, because the drugs classically used for preventing vasoconstriction, such as L-type channel-specific dihydropyridine antagonists, appear to potentiate, rather than inhibit, mineralocorticoid secretion.

### Acknowledgments

We are grateful to Mrs. L. Bockhorn, G. Dorenter, W. Dimeck, and M. Lopez for their excellent technical assistance.

### References

- Barrett PQ, Bollag WB, Isales CM, McCarthy RT, Rasmussen H 1989 Role of calcium in angiotensin II-mediated aldosterone secretion. *Endocr Rev* 10:496-518
- Spät A, Enyedi P, Hajnoczky G, Hunyady L 1991 Generation and role of calcium signal in adrenal glomerulosa cells. *Exp Physiol* 76:859-885
- Capponi AM, Python CP, Rossier MF 1994 Molecular basis of angiotensin II action on mineralocorticoid synthesis. *Endocrine* 2:579-586
- Fakunding JL, Chow R, Catt KJ 1979 The role of calcium in the stimulation of aldosterone production by adrenocorticotropin, angiotensin II, and potassium in isolated glomerulosa cells. *Endocrinology* 105:327-333
- Rossier MF, Krause K-H, Lew PD, Capponi AM, Vallotton MB 1987 Control of cytosolic free calcium by intracellular organelles in bovine adrenal glomerulosa cells: effects of sodium and inositol 1,4,5-trisphosphate. *J Biol Chem* 262:4053-4058
- Rossier MF, Capponi AM, Vallotton MB 1988 Inositol trisphosphate isomers in angiotensin II-stimulated adrenal glomerulosa cells. *Mol Cell Endocrinol* 57:163-168
- Balla T, Baukal AJ, Guillemette G, Morgan RO, Catt KJ 1986 Angiotensin-stimulated production of inositol trisphosphate isomers and rapid metabolism through inositol 4-monophosphate in adrenal glomerulosa cells. *Proc Natl Acad Sci USA* 83:9323-9327
- Burnay MM, Python CP, Vallotton MB, Capponi AM, Rossier MF 1994 Role of the capacitative calcium influx in the activation of steroidogenesis by angiotensin II in adrenal glomerulosa cells. *Endocrinology* 135:751-758
- Rohacs T, Bago A, Deak F, Hunyady L, Spät A 1994 Capacitative calcium influx in adrenal glomerulosa cells: possible role in angiotensin II response. *Am J Physiol* 267:C1246-C1252
- Matsunaga H, Yamashita N, Maruyama Y, Kojima I, Kurokawa K 1987 Evidence for two distinct voltage-gated calcium channel currents in bovine adrenal glomerulosa cells. *Biochem Biophys Res Commun* 149:1049-1054
- Rossier MF, Python CP, Capponi AM, Schlegel W, Kwan CY, Vallotton MB 1993 Blocking T-type calcium channels with tetrandrine inhibits steroidogenesis in bovine adrenal glomerulosa cells. *Endocrinology* 132:1035-1043
- Barrett PQ, Isales CM, Bollag WB, McCarthy RT 1991 Calcium channels and aldosterone secretion: modulation by potassium and atrial natriuretic peptide. *Am J Physiol* 261:F706-F719
- Rossier MF, Aptel HBC, Python CP, Burnay MM, Vallotton MB, Capponi AM 1995 Inhibition of low threshold calcium channels by angiotensin II in adrenal glomerulosa cells through activation of protein kinase C. *J Biol Chem* 270:15137-15142
- Rasmussen H, Isales CM, Calle R, Throckmorton D, Anderson M, Gasalla-Herraz J, McCarthy RT 1995 Diacylglycerol production, calcium influx, and protein kinase C activation in sustained cellular responses. *Endocr Rev* 16:649-681
- Barrett PQ, Ertel EA, Smith MM, Nee JJ, Cohen CJ 1995 Voltage-gated calcium currents have two opposing effects on the secretion of aldosterone. *Am J Physiol* 268:C985-C992
- Balla T, Varnai P, Hollo Z, Spät A 1990 Effects of high potassium concentration and dihydropyridine calcium channel agonists on cytoplasmic calcium and aldosterone production in rat adrenal glomerulosa cells. *Endocrinology* 127:815-822
- Horn R, Marty A 1988 Muscarinic activation of ionic current measured by a new whole cell recording method. *J Gen Physiol* 92:145-159
- Rae J, Cooper K, Gates P, Watsky M 1991 Low access resistance perforated patch recordings using amphotericin B. *J Neurosci Methods* 37:15-26
- Suzuki S, Kawakami K, Nishimura S, Watanabe Y, Yagi K, Seino M, Miyamoto K 1992 Zonisamide blocks T-type calcium channel in cultured neurons of rat cerebral cortex. *Epilepsy Res* 12:21-27
- Capponi AM, Rossier MF, Davies E, Vallotton MB 1988 Calcium stimulates steroidogenesis in permeabilized bovine adrenal cortical cells. *J Biol Chem* 263:16113-16117
- Rizzuto R, Simpson AWM, Brini M, Pozzan T 1992 Rapid changes of mitochondrial calcium revealed by specifically targeted recombinant aequorin. *Nature* 358:325-327
- Rizzuto R, Brini M, Murgia M, Pozzan T 1993 Microdomains with high calcium close to inositol trisphosphate-sensitive channels that are sensed by neighboring mitochondria. *Science* 262:744-747
- McCormack JG, Daniel RL, Osbaldeston NJ, Rutter GA, Denton RM 1992 Mitochondrial calcium transport and the role of matrix calcium in mammalian tissues. *Biochem Soc Trans* 20:153-159
- Python CP, Laban OP, Rossier MF, Vallotton MB, Capponi AM 1995 The site of action of calcium in the activation of steroidogenesis: studies in calcium-clamped bovine adrenal zona glomerulosa cells. *Biochem J* 305:569-576
- Fitzpatrick SC, McKenna TJ 1992 Evidence for a tonic inhibitory role of nifedipine-sensitive calcium channels in aldosterone biosynthesis. *J Steroid Biochem Mol Biol* 42:575-580
- Wu L, Wang R, Karpinski E, Pang PKT 1992 Bay K-8644 in different solvents acts as a transient calcium channel antagonist and a long-lasting calcium channel agonist. *J Pharmacol Exp Ther* 260:966-974
- Richard S, Diochot S, Nargeot J, Baldy-Moulinier M, Valmier J 1991 Inhibition of T-type calcium currents by dihydropyridines in mouse embryonic dorsal root ganglion neurons. *Neurosci Lett* 132:229-234
- Dunlap K, Luebke JI, Turner TJ 1995 Exocytotic calcium channels in mammalian central neurons. *Trends Neurosci* 18:89-98
- Sheng Z-H, Rettig J, Cook T, Catterall WA 1996 Calcium-dependent interaction of N-type calcium channels with the synaptic core complex. *Nature* 379:451-454
- Bezprozvanny I, Scheller RH, Tsien RW 1995 Functional impact of syntaxin on gating of N-type and Q-type calcium channels. *Nature* 378:623-626
- Allbritton NL, Meyer T, Stryer L 1992 Range of messenger action of calcium ion and inositol 1,4,5-trisphosphate. *Science* 258:1812-1815
- Capponi AM, Johnson EIM, Rossier MF, Lang U, Vallotton MB 1989 The calcium messenger system in angiotensin II-induced aldosterone production. In: Mantero F, Takeda R, Scoggins BA, Biglieri EG, Funder JW (ed) *The Adrenal and Hypertension: From Cloning to Clinic*. Raven Press, New York, vol 57:45-52
- Varnai P, Osipenko ON, Vizi ES, Spät A 1995 Activation of calcium current in voltage-clamped rat glomerulosa cells by potassium ions. *J Physiol* 483:67-78
- Aptel HB, Johnson EIM, Vallotton MB, Rossier MF, Capponi AM 1996 Demonstration of an angiotensin II-induced negative feedback effect on aldosterone synthesis in isolated rat adrenal zona glomerulosa cells. *Mol Cell Endocrinol* 119:105-111

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**